

Relation Between Serology of Meat Juice and Bacteriology of Tonsils and Feces for the Detection of Enteropathogenic *Yersinia* spp. in Pigs at Slaughter

Inge Van Damme,¹ Gerty Vanantwerpen,¹ Dirk Berkvens,² and Lieven De Zutter¹

Abstract

The association between positive serology and culture detection of *Yersinia* spp. in individual pigs was determined. Pieces of diaphragm from 370 pig carcasses were collected for serological analysis, and tonsils and feces of the same carcass were collected for bacteriological analysis. Detection of anti-*Yersinia* antibodies in meat juice samples was done using an indirect enzyme-linked immunosorbent assay (ELISA) based on Yops (*Yersinia* outer proteins). Tonsils and feces were tested for the presence of enteropathogenic *Yersinia* spp. by direct plating on cefsulodin–irgasan–novobiocin agar plates. Of the 370 meat juice samples, 241 (65.1%) gave a positive serological reaction using a cutoff value of 20%. Enteropathogenic *Yersinia* spp. (*Yersinia enterocolitica* serotype O:3 and *Yersinia pseudotuberculosis*) were found in tonsils of 161 pigs and feces of 30 pigs. Recovery of enteropathogenic *Yersinia* from the tonsils was highly correlated with positive serotiters, whereas no correlation was found between serology and fecal excretion. Results demonstrated that serology has an acceptable sensitivity, but a relatively low specificity for the rapid detection of enteropathogenic *Yersinia* spp. in tonsils of pigs at slaughter.

Introduction

ENTEROPATHOGENIC *YERSINIA* spp. (virulent strains of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*) are foodborne pathogens of the gastrointestinal tract, but have a strong tendency to extraintestinal spread under certain host conditions (Bottone, 1997; Long *et al.*, 2010). All pathogenic *Yersinia* harbor a virulence plasmid (pYV), which encodes several yops (*Yersinia* outer proteins) that modulate the immune system and allow *Yersinia* to survive and replicate extracellularly within lymphoid tissues of the host (Heesemann *et al.*, 2006).

Y. enterocolitica is mainly transmitted to humans through the consumption of contaminated pork (Fosse *et al.*, 2008; Rosner *et al.*, 2012). A high proportion of pigs at slaughter are infected with this pathogen, mainly bioserotype 4/O:3 (Bucher *et al.*, 2008; Ortiz Martínez *et al.*, 2009). *Y. pseudotuberculosis* is also found in slaughter pigs, though to a lesser extent than *Y. enterocolitica* (Ortiz Martínez *et al.*, 2009). The pathogens are present in the oral cavity (particularly tonsils and tongue), lymph nodes, and the gastrointestinal tract. As pigs carry *Y. enterocolitica* without showing any symptoms, traditional meat inspection fails to detect infected

pigs. During slaughter and dressing operations, enteropathogenic *Yersinia* spp. may spread to the rest of the carcass (Borch *et al.*, 1996; Laukkanen *et al.*, 2008, 2009).

To ensure an effective control of the main biological hazards on pork carcasses, preventive measures and controls should be applied both on-farm and at abattoir level. As carcass contamination with enteropathogenic *Yersinia* spp. depends on the contamination status of pigs delivered to the slaughterhouse (Laukkanen *et al.*, 2008, 2009), incoming pig batches should be differentiated with respect to the risk they pose for public health (EFSA, 2011). Bacteriological monitoring of tonsils provides the best indication for the presence of *Yersinia* in slaughter pigs (Nesbakken *et al.*, 2006), albeit time consuming and labor intensive (Fredriksson-Ahomaa and Korkeala, 2003). In contrast, serological screening of antibodies is a more convenient and faster alternative, given that the correlation between antibody titers and culture is high. Hence, the current study was conducted to assess the agreement between serological and bacteriological tests at animal level under field conditions. Accordingly, *Yersinia* antibodies in meat juice and the presence of enteropathogenic *Yersinia* spp. in tonsils and feces of pigs at slaughter were determined to estimate the relative sensitivity and specificity

¹Department of Veterinary Public Health and Food Safety, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium.

²Unit of Veterinary Epidemiology, Department of Biomedical Sciences, Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium.

of the serology test for predicting the presence of *Yersinia* in slaughter pigs.

Materials and Methods

Sample collection

The tonsils, feces, and 10 g of diaphragm muscle of 370 pig carcasses, originating from 194 farms (1–14 pigs per farm), were sampled during a 1-year period in 10 pig slaughterhouses in Belgium. Tonsils were excised aseptically after removal of the plug set and put in a sterile plastic bag. The whole intestinal tract was collected immediately after evisceration and the rectum was closed with threads, after which it was excised and put in a plastic bag. All samples were transported to the laboratory under refrigerated temperatures.

Serological analysis

Diaphragm muscles were frozen (–20°C) immediately upon arrival for 24 h and then thawed at 4°C for 24 h, after which the meat juice was collected and stored at –20°C until analysis. Samples were examined for antibodies against pathogenic *Yersinia* using YOPSCREEN Pig enzyme-linked immunosorbent assay (ELISA) (Labor Diagnostik, Leipzig, Germany) according to the manufacturer's instructions. The presence of antibodies against Yops (*Yersinia* outer proteins) of plasmid-bearing *Yersinia* was determined by measuring the optical density (OD) at 450 nm. Activity values (OD%) were calculated based on OD values, relative to the mean OD values of positive and negative controls (OD_{pos} and OD_{neg}, respectively).

$$\text{Activity value (OD\%)} = \frac{OD_{\text{sample}} - OD_{\text{neg}}}{OD_{\text{pos}} - OD_{\text{neg}}}$$

Culture detection of enteropathogenic *Yersinia* spp.

Tonsils and feces were examined for enteropathogenic *Yersinia* spp. by direct plating on cefsulodin–irgasan–novobiocin (CIN) (Bio-Rad, Nazareth, Belgium) agar plates (Van Damme *et al.*, 2010). In short, 10 g of tissue was homogenized 1/10 (wt:wt) and 1 mL of homogenate was spread plated on two agar plates. Additionally, 100 µL was inoculated on a CIN agar using a spiral plate machine (Eddie Jet, IUL Instruments, Barcelona, Spain). Human pathogenic *Y. enterocolitica* isolates were confirmed using polymerase chain reaction (PCR) assays targeting the *ail*, *ystA*, *virF*, *rfbC*, and *per* genes as described earlier (Van Damme *et al.*,

2013). *Y. pseudotuberculosis* isolates were identified using a PCR assay targeting the *inv*-gene (Nakajima *et al.*, 1992).

Statistical analyses

Statistical analyses were performed using Stata/MP 12.1 (StataCorp, 2011). The relative sensitivity and specificity of the ELISA test using four different cutoff values (10%, 20%, 30%, and 40%) were estimated relative to culture of enteropathogenic *Yersinia* spp. from the tonsils and feces as criterion standard. For the remaining analyses, the proposed cutoff value of the manufacturer of 20% was used as cutoff value for a positive result. The proportion of positive pigs by each method was compared using a mixed-effects logistic regression analysis, including the individual pig and farm as random effects. The association between serology (positive/negative) and culture results (presence/absence) was determined using a mixed-effects logistic regression, including the interaction between culture results from tonsils and feces. Farm was included as a random effect to account for clustering of pigs within a farm.

Based on bacteriological results, animals were classified in four different groups (group 1: tonsils and feces negative, group 2: tonsils negative and feces positive, group 3: tonsils and feces positive, and group 4: tonsils positive and feces negative). The seroprevalences within these groups were compared using a mixed-effects logistic regression including group as factor variable and farm as random effect. The activity values of seropositive pigs between groups were compared using a bootstrap median regression.

The association between lairage time (in hours) and bacteriological/serological results were analyzed using random-effects logistic regressions, including slaughterhouse and farm as random effects whenever necessary. Only pigs for which the lairage time was known were used for the analysis ($n=321$).

Results

The sensitivities and specificities of serology relative to culture examination of tonsils and feces are shown in Table 1. Compared to bacteriology of tonsils, the relative sensitivity and specificity of the ELISA test varied from 94.4% to 83.2% and from 41.6% to 69.9%, depending on the applied cutoff value. The relative sensitivity and specificity were lower when serology was compared to fecal excretion as criterion standard. Using a cutoff value of 20%, the sensitivities of the ELISA test were 91.9% and 63.3% compared to carriage of *Yersinia* in tonsils and excretion in feces as reference,

TABLE 1. RELATIVE SENSITIVITIES AND SPECIFICITIES OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY TEST FOR DIFFERENT CUTOFF VALUES, BASED ON BACTERIOLOGICAL RESULTS OF TONSILS, FECES, AND BOTH

Cutoff value	Tonsils		Feces		Both ^a	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
10%	94.4	41.6	73.3	25.9	93.0	42.2
20%	91.9	55.5	63.3	34.7	89.5	55.8
30%	88.8	62.2	63.3	40.3	86.6	62.8
40%	83.2	69.9	56.7	47.1	80.1	69.9

^aBoth: tonsils, feces, or at least one test is positive.

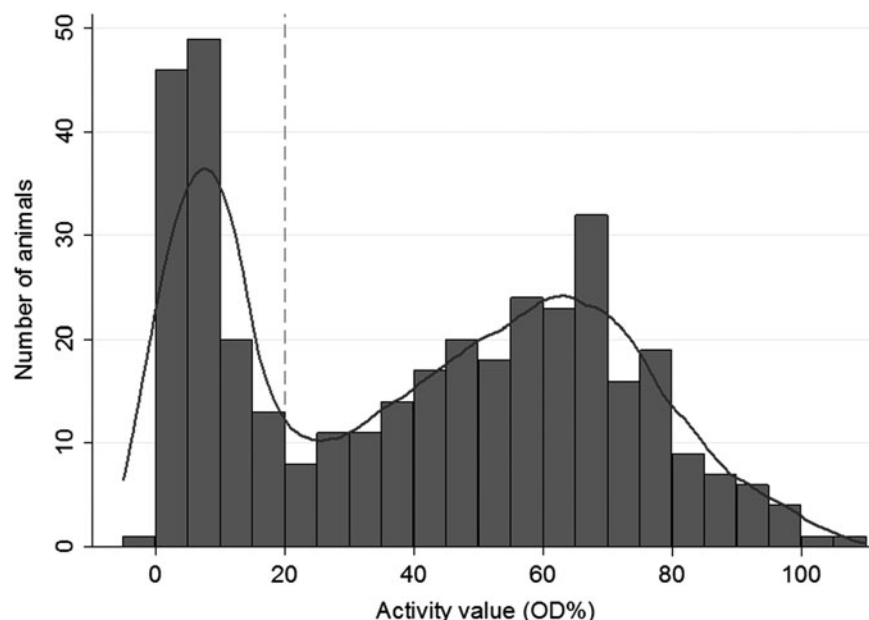


FIG. 1. Frequency distribution of the activity values (optical density [OD]%) from meat-juice samples of 370 slaughter pigs. The solid line represents an Epanechnikov Kernel density distribution.

respectively. The relative specificities were 55.5% and 34.7%, respectively.

The histogram of the activity values in Figure 1 shows a bimodal distribution of the serological data. Ninety-six samples (25.9%) had an activity value below 10%. Thirty-three samples (8.9%) had an activity value between 10% and 20%, and 19 samples (5.1%) between 20% and 30%. In total, 65.1% of pigs were seropositive based on a cutoff value of 20%. The average OD% of seronegative and seropositive animals was 7.8 ± 4.5 ($n=129$) and 58.6 ± 18.7 ($n=241$), respectively.

In total, enteropathogenic *Yersinia* spp. were recovered from 161 tonsils (43.5%) and 30 fecal samples (8.1%). Overall, 171 pigs (46.2%) carried enteropathogenic *Yersinia* spp. in the tonsils and/or excreted them in the feces (Table 2). *Y. enterocolitica* serotype O:3 was detected in the tonsils and feces of 159 and 30 pigs, respectively, whereas *Y. pseudotuberculosis* was recovered from 3 tonsils only, of which 1 pig had a concurrent *Y. enterocolitica* infection. The number

of pigs showing serological evidence of infection (based on a cutoff of 20%) was significantly higher than the number of pigs carrying enteropathogenic *Yersinia* in the tonsils ($p < 0.001$), both tests being significantly higher than the rate of pigs excreting enteropathogenic *Yersinia* spp. in the feces ($p < 0.001$).

From the 161 pigs that carried enteropathogenic yersiniae in the tonsils, 148 (91.9%) tested serologically positive using a cutoff value of 20% (Table 2). Out of the 30 pigs that excreted the pathogens in the feces, 19 (63.3%) were seropositive. Eighty-eight (23.8%) of the tested pigs were serologically positive but showed no evidence of a current infection based on bacteriological results. A strong positive association was found between serology and the presence of enteropathogenic yersiniae in tonsils (adjusted odds ratio [OR]=64.37, $p < 0.001$), whereas no relation was found for fecal samples (adjusted OR=0.94, $p=0.946$). Moreover, a negative interaction between tonsils and feces was found, which had borderline significance (adjusted OR=0.08, $p=0.059$).

On the basis of bacteriological results from tonsils and feces, pigs were allocated into four groups (Table 2). The seroprevalence increased over the different groups, and was highest for pigs that carried *Yersinia* in the tonsils but did not excrete the pathogens in the feces in levels detectable by direct plating (> 10 colony-forming units [CFU]/g) ($p=0.006$). The distribution of the activity values within each bacteriological group is shown in Figure 2. In group 1 (tonsils and feces negative), the majority of pigs were seronegative, generally having an activity value below 10%. The activity value of most seropositive pigs in this group was situated around 50%. Contrarily, most pigs in group 4 (tonsils positive and feces negative) were seropositive, showing an activity value around 65%–70%. The median activity values in seropositive pigs were significantly higher in groups 3 and 4 than in group 1 ($p < 0.001$).

The average holding time of the sampled pigs was 2.82 h, with a standard error of 0.13 h. The holding time of pigs that

TABLE 2. BACTERIOLOGICAL AND SEROLOGICAL RESULTS OF 370 PIGS AT SLAUGHTER

Group	Bacteriology			Serology (cutoff 20%)		
	Tonsils	Feces	n ^a	Positive	Negative	% ^b
1	–	–	199	88	111	44.22
2	–	+	10	5	5	50.00
3	+ ^c	+	20	14	6	70.00
4	+	–	141	134	7	95.04
Total			370	241	129	65.14

^aTotal number of samples within each group.

^bSeroprevalence within each group.

^cPositive for pathogenic *Yersinia enterocolitica* and/or *Y. pseudotuberculosis* (based on direct plating on cefsulodin–irgasan–novobiocin agar; detection limit of 10 colony-forming units/g).

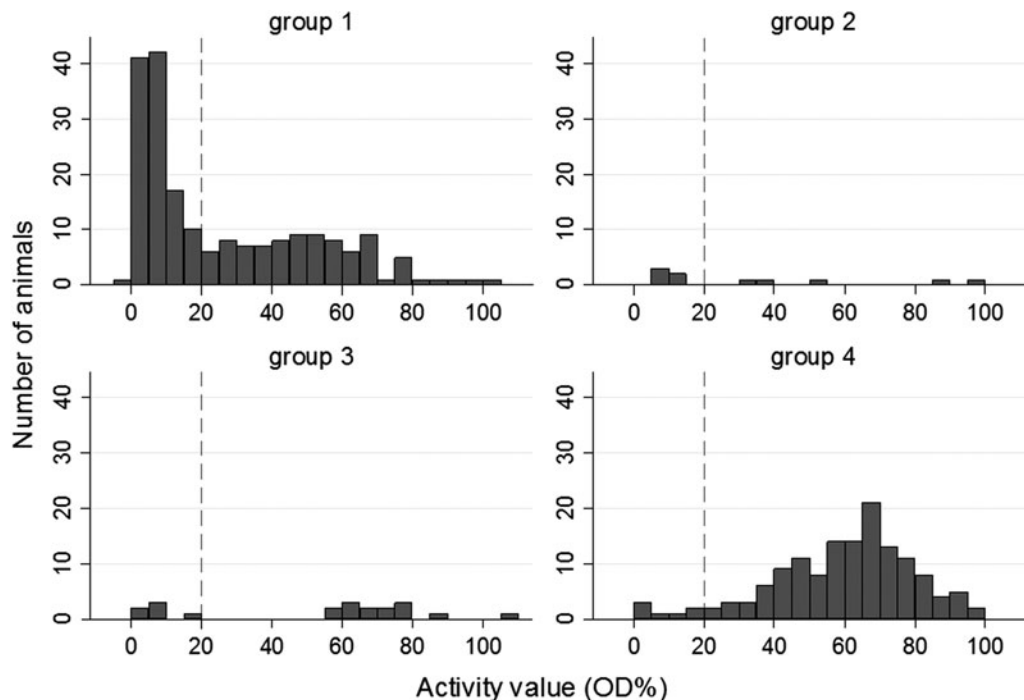


FIG. 2. Frequency distribution of activity values (optical density [OD]%) of meat juice samples in different groups based on bacteriological results (tonsillar carriage and fecal excretion). Group 1: tonsils and feces negative; group 2: tonsils negative, feces positive; group 3: tonsils and feces positive; group 4: tonsils positive, feces negative.

excreted yersiniae in the feces (3.95 ± 0.57 h) was significantly higher than that of pigs that did not excrete yersiniae (2.73 ± 0.13 h) ($p=0.015$). No association was found between lairage time and tonsillar carriage ($p=0.311$) nor between lairage time and the serological response ($p=0.908$).

Discussion

This study evaluated the use of serology as an alternative to bacteriological testing to detect *Yersinia* infections in pigs. Antibodies were detected in 65% of pigs, while 43% carried enteropathogenic yersiniae in the tonsils and only a small proportion of animals excreted the pathogens in the feces (8%). The ELISA test showed a high correlation and an acceptable sensitivity but limited specificity when compared to bacteriological culture of tonsils as reference.

In contrast to culture examination, a serological reaction is a historical measure of infection, which inherently limits the specificity of serology when compared to bacteriology. Although some animals may test negative for enteropathogenic *Yersinia* in the tonsils and feces after a certain period, the serological reaction remains positive for at least 70 days after infection (Nielsen *et al.*, 1996). The activity values of seropositive pigs were lower in culture-negative animals than in pigs that carried yersiniae in the tonsils, which may represent the decrease in antibodies after elimination of infection. Nevertheless, besides the biological differences between serology and bacteriology, a high association was found between seropositivity and isolation of *Yersinia* from the tonsils, whereas no association was found between serology and fecal *Yersinia* excretion. Similarly, von Altrock *et al.* (2006) analyzed blood and feces from 900 pigs in 30 fattening-pig herds shortly before slaughter and no correlation

was found between serological and culture results. Pigs can carry the pathogen in tonsils for several months, whereas fecal excretion declines more rapidly (Nielsen *et al.*, 1996; Virtanen *et al.*, 2012). However, the time period after experimental infection in which *Yersinia* are detectable in the feces is influenced by the isolation method used, and possibly results from a decline in numbers over time (Nielsen *et al.*, 1996). As only direct plating was used in the present study, only animals excreting higher numbers (> 10 CFU/g) were detected, which may indicate that they were relatively more recently infected. Recently infected animals (less than 2–3 weeks before slaughter) are less likely to have seroconverted, which might partly explain the low relative sensitivity of the serological test to detect excretion of enteropathogenic *Yersinia* spp. in the feces in the present study.

Pathogenic *Y. enterocolitica* can be recovered from tonsils and intestines within hours after oral inoculation of pigs (Thibodeau *et al.*, 1999), whereas seroconversion is observed after 12–19 days of experimental infection (Nielsen *et al.*, 1996). Similarly, Nesbakken *et al.* (2006) followed pigs on two naturally infected *Yersinia*-positive farms and observed that contamination of tonsils and feces started around the same time, whereas seroconversion occurred about 2 weeks later. In the present study, only a small part of the tested pigs (18/370, 5%) were serologically negative while they carried and/or excreted pathogenic *Yersinia* in the tonsils and feces, which may indicate a recent infection. Initial infection may have taken place on-farm during the final stage of the fattening period, but also during transport or lairage at the abattoir.

The average lairage time for all pigs was almost 3 h, which is sufficiently long for *Yersinia* to colonize the tonsils and intestines under experimental conditions (Thibodeau *et al.*,

1999). Nevertheless, only for fecal excretion a significant association was found with longer lairage times. For every increase in holding time with 1 h, the odds of finding enteropathogenic *Yersinia* spp. in the feces increased with a factor 1.20. This association may indicate that the risk of infection (and fecal excretion) increases with longer lairage times or that fecal excretion increases with increasing holding times (e.g., due to stress).

Several factors may influence serological results, such as the applied antigen, sample type, and cutoff value. The present study used muscle fluid to detect *Yersinia* antibodies in pigs at slaughter as it is a faster and easier alternative compared to serum. The ELISA test contains Yop antigens, so that both human pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* antibodies are detected. On the other hand, ELISA assays using specific antigens against *Y. enterocolitica* serotype O:3 (Nielsen *et al.*, 1996) may detect antibodies against the most common pathogenic serotype in pigs, but not the more rare *Y. enterocolitica* serotypes and *Y. pseudotuberculosis*. Nevertheless, as in most European countries, *Y. enterocolitica* serotype O:3 predominates in pigs (Bucher *et al.*, 2008; Ortiz Martínez *et al.*, 2009; Van Damme *et al.*, 2010), this may have a small impact on the final outcome.

The activity values showed a bimodal distribution with modes at about 5% and 65%, which may represent the seronegative and seropositive population, respectively. Accordingly, this distribution would suggest that the cutoff value for the ELISA test should be between 20% and 30% and indicates that the proposed cutoff value of 20% of the manufacturer corresponds with the field data.

Besides test-related factors, biological aspects such as age of the pigs and prevalence differences (e.g., between regions and farms) may also affect the serological outcome. Nevertheless, despite many influencing factors, the proportion of slaughter pigs with *Yersinia* antibodies is usually very similar among different studies, as the seroprevalence found here (65%) agrees with the 66% in Canada (Thibodeau *et al.*, 2001) and 64% found in fattening pigs just before or during slaughter in Germany (von Altröck *et al.*, 2011).

Conclusions

Serology of meat juice of pigs at slaughter correlates well with the carriage of pathogenic *Yersinia* in tonsils, whereas serological findings show no relation with *Yersinia* excretion in the feces. The meat juice ELISA can thus be used as an indicator for *Yersinia* carriage at the individual level, though the low epidemiologic specificity may result in many false-positive reactions.

Acknowledgments

The authors thank Annelies Wachtelaer and Anne-Mie Terryn for the technical assistance. The slaughterhouses are kindly acknowledged for their cooperation in sampling. This study was funded by a doctoral grant from the Special Research Fund (BOF, Bijzonder Onderzoeksfonds, BOF09/24J/064) of Ghent University, Belgium.

Disclosure Statement

No competing financial interests exist.

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- Address correspondence to:
Inge Van Damme, DVM, PhD
Department of Veterinary Public Health
and Food Safety
Faculty of Veterinary Medicine,
Ghent University
B-9820 Merelbeke, Belgium
E-mail: inge.vandamme@ugent.be